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THE EFFECT OF THYROXINE ON MYOCARDIAL HIGH ENERGY PHOSPHATES

Wayne DeWitt Brenckman, Jr.

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THE EFFECT OF THYROXINE ON MYOCARDIAL HIGH ENERGY PHOSPHATES

By

Wayne DeWitt Brenckman, Jr.

A thesis submitted to the Faculty of the
Yale University School of Medicine in candidacy
for the degree Doctor of Medicine.

Department of Physiology
Yale University School of Medicine
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CONTENTS

ABBREVIATIONS 1

INTRODUCTION 2

(1) Mechanisms of muscle failure 3

A: Impairment of the metabolic supply 4

B: "Failure" without obvious impairment of oxidative metabolism 5

C: "Failure" with alteration in the physical properties of the contractile proteins 7

(2) The thyroid hormones 7

MATERIALS AND METHODS 11

EXPERIMENTAL RESULTS 22

(1) Effect of the gas mixture for respiration 22

(2) The total acid soluble phosphate fraction 23

(3) The ATP fraction 24

(4) The ADP fraction 25

(5) The AMP fraction 25

(6) The CP fraction 26

(7) The Pi fraction 26

(8) Recovery percentages 27

(9) Water content of the fresh myocardial tissue 27

(10) Summary of the experimental results 28

DISCUSSION 29

SUMMARY AND CONCLUSIONS 36

APPENDIX I: Composition of reagents and solvents for chromatography 38

APPENDIX II: The colorimetric determination of phosphorus . . . 39

APPENDIX III: Calculation of the acid soluble phosphate concentrations 42

REFERENCES 45

ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
IMP	Inosine monophosphate
CP	Creatine phosphate, phosphocreatine
Pi	Inorganic phosphate
DNP	2, 4-dinitrophenol
TASP	Total acid soluble phosphate (s)

INTRODUCTION

At the present time, the overwhelming weight of experimental evidence indicates that the source of energy for muscle contraction comes, directly or indirectly, from the splitting of certain "high energy" phosphate bonds. There is no reason to believe that the metabolic pathways supplying these energy rich phosphate compounds differ in any significant way from the well-known glycolytic and aerobic pathways of other body cells. The unique feature of muscle tissue is that it possesses a protein system capable of transforming this chemical energy into mechanical energy by contraction. Numerous chemical analyses of myocardial tissue from many different species have shown that by far the largest quantity of high energy phosphate bonds, of the nature suitable for utilization by the contractile system, occurs in the adenosine triphosphate (ATP) and creatine phosphate (CP) fractions of the muscle (Wollenberger, 1958; Fleckenstein et al., 1959).

The exact mechanism whereby the energy of the ATP and CP is coupled to the contractile proteins is not entirely clear. If ATP or CP were directly hydrolyzed during a single muscle twitch, one should expect, momentarily at least, to observe a reduction in the concentration of cellular ATP or CP and an increase in the inorganic phosphate (Pi) fraction. Experiments to demonstrate such an effect have yielded equivocal results; Fleckenstein et al. (1954a, 1954b) and Mommaerts (1954, 1955) were unable to detect any breakdown of either ATP or CP after a single, rapidly interrupted, muscle twitch, but did find a small increase in the Pi fraction. This set off an

intense search for a compound, "XP", which was assumed to be the immediate phosphate donor (Davies et al., 1959). Mommaerts (1961) found that the increase in Pi during a single twitch of frog sartorius muscle was accompanied by the formation of an equivalent amount of creatine in the muscle; however, no consistent decrease of CP occurred and Mommaerts was unable to account for these findings.

In contrast to the situation prevailing during a single twitch, Fleckenstein et al. (1960) observed a significant decrease in the CP fraction with a concomitant increase in the Pi following a series of twitches, or during periods of brief tetanic stimulation of the frog rectus muscle. Under these conditions, no decrease in ATP was noted. The most widely accepted explanation for this, summarized by Mommaerts (1961), is that the ATP and CP are in equilibrium during the steady state, and that increased demands for high energy phosphate hydrolysis, imposed by an increased work requirement, will not reflect the additional breakdown of ATP, since the liberated ADP is rapidly rephosphorylated by the CP. This is the so-called Lohmann reaction (Lohmann, 1934).

(1) MECHANISMS OF MUSCLE FAILURE

For the efficient performance of work by a muscle a fine synchronization of several systems is required. It is conceivable that a muscle can "fail" because of a diminished rate of formation or supply of ATP or CP to a normal contractile apparatus. It is equally possible that the metabolic pathways could be capable of furnishing adequate amounts of these energy rich phosphates to intact contractile proteins, but that their utilization might be rendered less efficient.

Finally, there may be "failure" due to some aberation in the physical structure of the contractile protein. Current opinion holds that reasonably clear-cut examples of the first two situations do exist (Furchgott and Lee, 1961).

A: Impairment of the metabolic supply. An example of metabolic myocardial failure is seen in the case of anoexmia or anoxia produced at the cellular level by chemical means. Numerous observations (e.g. Fleckenstein et al., 1959, p. 243) indicate that there is no quicker way to deplete the myocardium of CP and ATP than to interrupt the coronary circulation. Artificial respiration with a mixture of 4% oxygen in nitrogen causes the same end result after a slightly longer latent period (Fleckenstein et al., 1959). Similar effects have been produced in situ by the intravenous injection of 2,4-dinitrophenol (DNP) in the rat (Fleckenstein et al., 1959), by the use of DNP in the dog heart-lung preparation (Fawaz and Tutunji, 1957), and by poisoning the guinea pig ventricle in a heart-lung preparation with phenylbutazone (Hockrein and Döring, 1958). All of these situations are characterized by an early, rapid, and marked fall in the levels of CP, together with a decrease in the contractile strength of the preparation as compared with its resting value. The decrease in ATP content of these preparations proceeds more slowly and does not begin until a significant amount of the CP has disappeared. In Furchgott and Lee's (1961) in situ myocardial preparations from the asphyxiated guinea pig, resumption of respiration resulted in rapid reconstitution of the CP fraction to nearly control levels, while the ATP reaccumulated more slowly. Contractile strength recovered concomitantly with the

resynthesis of the CP and was nearly normal even when the ATP level was considerably depressed. In all of the metabolically damaged preparations mentioned above, the CP levels were observed to decrease to 50 to 90%, and the contractile strength to 65 to 90%, of their control levels, while the ATP decrease was of the order of 7 to 50%. A clinical example of a derangement in oxidative metabolism with concomitant myocardial failure is associated with thiamine deficiency and beriberi heart disease. In none of these metabolic disorders is the use of cardiac glycosides attended by significant improvement (Furchgott and Lee, 1961).

B: "Failure" without obvious impairment of oxidative metabolism.

Many instances have now been recorded in which myocardial tissue in experimental "failure" showed no decrease of the ATP or CP content as compared with the "pre-failure" levels. Such findings were observed in Wollenberger's (1947) dog heart-lung preparation, in the *in situ* dog hearts of Olson and Piatneck (1959) rendered incompetent by tricuspid valve avulsion and pulmonary artery constriction, in the "spontaneously" failing guinea pig left atrium *in vitro* (Furchgott and de Gubareff, 1958), and the "spontaneously" failing cat papillary muscle *in vitro* (Lee et al., 1960a). One criticism which may be leveled at all of these studies, concerns the moot point of whether or not this represents failure of the heart. A case of particular interest is the *in situ* guinea pig preparation of Furchgott and Lee (1961) which underwent chronic failure secondary to aortic constriction. A rather pronounced decrease from control levels of CP (-54%), ATP (-24%), and ADP (-40%) was observed in the myocardial preparations of

these animals. The signs present in these animals included "cardiac hypertrophy, pulmonary edema, elevated venous and right ventricular pressures, and elevated left diastolic pressures". The data for these guinea pigs are reminiscent of those observed in the myocardia of animals who suffer some primary metabolic derangement, and indeed, when the mitochondria from these chronically failing guinea pig hearts were examined, they were found to have an oxidative phosphorylation efficiency (measured by P:O ratios) of 30 to 40% below those from normal animals. Thus, the decrease in high energy phosphates in these animals may be due in part to an impairment of the metabolic apparatus to effect their synthesis.

In another series of experiments, Furchgott and Lee (1961) studied guinea pig atria and cat papillary muscles in vitro, in which contractile strength was altered by the addition of certain drugs to the bathing medium, or which were allowed to undergo "spontaneous" failure prior to the addition of inotropic agents. There was usually no alteration in the concentrations of the high energy phosphates from the control levels, either during "failure" or upon "recovery from failure" with the inotropic agents. Briefly, their results showed that levels of ATP and CP were unaffected by increasing the contractile strength with a medium of high calcium content, with low concentrations of epinephrine, and with cardiac glycosides at concentrations giving maximum inotropic effects. Also, no effect on myocardial ATP or CP content was observed after decreasing the contractile strength with a medium low in calcium, with acetylcholine, with ryanodine, or by reducing the frequency of contraction from 60 these animals. The [illegible]

hypertrophy, [illegible]

pressures, [illegible]

these [illegible]

[illegible]

were [illegible]

were [illegible]

effects [illegible]

normal [illegible]

[illegible]

to 6 per minute. Cardiac glycosides used in toxic doses in these preparations did cause a decrease in ATP and CP, the decrease in CP being greater than the decrease in the ATP. However, Lee et al. (1960b) have shown that mitochondria obtained from hearts poisoned with toxic doses of cardiac glycosides have an impaired capacity for oxidative phosphorylation, and again the question of a decrease in high energy phosphates secondary to failure of synthesis must be raised. In general it would appear that the cardiac glycosides are most effective in relieving those types of failure resulting from inefficient utilization of otherwise normal quantities of energy rich phosphates.

C: "Failure" with alteration in the physical properties of the contractile proteins. There is considerable evidence (Holland and Klein, 1960; Olson et al., 1961) that the contractile proteins of the myocardium are altered in chronic congestive heart failure. Such hearts have shown decreased actomyosin concentrations, as well as the presence of a myosin with altered physical properties. In addition, there is reduced contractility of glycerol extracted fibers from hearts in congestive failure. It is difficult to say, however, whether these changes are primary or secondary to the cardiac insufficiency. The weight of opinion at the present time seems to support the latter view.

(2) THE THYROID HORMONES

The thyroid hormones, whose biochemical actions have been exhaustively reviewed by Hock (1962), can cause pronounced changes in cardiac function, and the heart failure of thyrotoxicosis is well known to the clinician. Thyroxine, in large dosages, is capable of

"uncoupling" oxidative phosphorylation in both intact (Lardy and Maley, 1954) and fragmented (Bronk, 1958) mitochondria in vitro. As Hoch points out, there is now good reason to believe that at the physiologic levels of thyroxine found in euthyroid animals, the action is not to uncouple, but rather to "loosely couple", oxidative phosphorylation -- a process which implies an increase in oxidative rate at normal phosphorylatory efficiency (P:O ratio), the net result of which is to increase the cellular quantities of ATP.

A number of analyses of both muscular and non-muscular tissue have been conducted in an attempt to evaluate the effect of hyperthyroidism on cellular high energy phosphate concentrations. Between 1933 and 1939 there appeared three short articles in the German literature in which young guinea pigs (Mattonet, 1933; Berg, 1937) or rats (Schumann, 1939) were given massive doses of thyroxine parenterally (ca. 1 mg per animal per day) for periods of 4-11 days and the total acid soluble phosphate fractions (TASP), "phosphagen" (now regarded as being synonymous with CP for mammalian cells) fractions, "acid labile" phosphate fractions, and inorganic phosphate fractions determined. The results purported to show a decrease in both the labile nucleotide-P and the phosphagen (CP) fractions. Actually, the precautions taken in obtaining these hearts for analysis were so meager that anoxia must have overriden all other effects. The control values of Pi in these experiments were three to five times as high as those reported in recent years, while the CP fractions were less than half of what one would expect.

Bertolini and Quarto di Palo (1956), in a brief paper that gives no experimental details, state that the ATP and CP content of the rat

ventricle are both decreased after daily injection of 10 micrograms of thyroxine per day for 10 and 15 day periods; indeed, they found not even a trace of CP after 10 days. While their control values of ATP were among the lowest recorded in the literature, the control levels of Pi were enormously high and accounted for 48% of their TASP. In fact, in their normal animals they accounted for 100% of the acid soluble-P in just three fractions--the ATP, CP, and Pi.

Conflicting reports of the effect of thyroxine upon liver high energy phosphates have appeared in the literature: Chatagner and Gautheron (1960) found this hormone to strongly depress the concentration and content of ATP in rat liver, whereas Chilson and Sachs (1959) were unable to detect a significant action on guinea pig liver ATP.

Finally, a great deal of caution should be exercised when relating the results of *in vitro* studies to the situation *in vivo*. In a recent series of experiments, Gerlach and Lübben (1959) observed that pigeon erythrocytes incubated *in vitro* with thyroxine (1×10^{-4} M) suffered a large breakdown of ATP. In contrast, the daily injection of thyroxine in doses sufficient to bring the birds to the point of death from general cachexia, failed to alter the erythrocyte ATP levels.

Analysis of myocardial tissue for high energy phosphates is not only a long, time-consuming affair, but--and it is only within the last ten years or so that this has been fully appreciated--a difficult one. The earlier literature values were often grossly inaccurate because of failure to appreciate the need for adequate oxygenation of the preparation and rapid freezing of the tissue *in situ*. Wollenberger et al. (1960a, 1960b) have shown, and Fawaz and Manoukian (1962) have

confirmed, that rapid excision of the heart from an animal with subsequent immersion in liquid nitrogen or isopentane at -150° does not reveal as high concentrations of the very labile CP as does _in situ_, "instantaneous", freezing of myocardium between aluminum blocks precooled in liquid nitrogen. The newer techniques of separation and identification that have become available in the last decade, such as the paper chromatographic separation of the adenine and guanine nucleotides (Fleckenstein et al. 1953, 1955), and the paper chromatographic separation of CP and Pi (Gerlach and Janke, 1958), afford a degree of specificity and accuracy unapproachable with the older methods of hydrolysis for "X" minutes in mineral acids with the determination of the liberated phosphorus (e.g. the method of Lohmann, 1928).

Because of the great interest attending the _in vivo_ action of thyroxine on the myocardium, and because techniques are now available which allow the simultaneous determination of ATP, ADP, AMP, CP, and Pi with heretofore unatainable accuracy, experiments were carried out on the apical ventricular myocardium of guinea pigs receiving large, but not massive, amounts of thyroxine for a two week period. As the experimental techniques used have not been described in the English literature, and in some respects have been extensively modified from their original German descriptions, they will be related in some detail in Appendices I-III.

The question posed; "Does the hyperthyroid myocardium suffer an alteration in its high energy phosphate metabolism?" and, if so, "can these changes be accounted for on the basis of an 'uncoupling' or 'loose coupling' of oxidative phosphorylation?"

MATERIALS AND METHODS

The experimental procedures used in these experiments will be discussed in five stages: (1) the treatment of the guinea pigs with thyroxine and the operative removal of the hearts, (2) the extraction of the acid soluble phosphates from the myocardium, (3) paperchromatographic separation and identification of the individual phosphorus containing fractions, (4) quantitative measurement of the chromatographically separated compounds by a determination of phosphorus content, and (5) calculation of the concentrations of these compounds per gram of apical myocardium.

(1)

A total of twenty male guinea pigs, weighing between 450 and 800 grams were used. Of these, eleven (designated A1 through A11) served as controls and received no thyroxine or other medication (except anesthesia) prior to removal and analysis of their hearts. The remaining nine (T1 through T9) were similar to the controls in all respects, but in addition received daily intraperitoneal injections of 80-100 micrograms of L-sodium thyroxine (Nutritional Biochemicals Corp., Cleveland, Ohio) for periods of 14 to 18 days. The thyroxine was dissolved in one or two drops of 0.1N NaOH and immediately diluted with isotonic saline to a volume between 50 and 100 ml such that one milliliter of solution contained 50 micrograms of the hormone. All animals receiving thyroxine were started at an initial dosage level of 100 micrograms/kg of body weight/day. The animals were weighed every two or three days, the first detectable weight loss usually occurring on the third day of treatment. If subsequent weighings

revealed a weight loss of greater than 20 gm in a two day period, the dosage was reduced to 90 micrograms/kg/day, or even to 80 micrograms in several very sensitive animals. Weight loss was progressive until about day 9 to 12 of the regimen, at which point a minimum weight was observed. After this, despite the same dosage schedule, the animals showed a slight but progressive weight gain; at the time of sacrifice (day 14-18) they had usually regained about one half of the initial loss.

To obtain the hearts for analysis the animals were weighed and then anesthetized with intraperitoneal sodium pentobarbital (Nembutal, Abbott, 27-30 mg/kg of body weight). The animals were strapped to a dissecting board, a tracheostomy was performed, and the chest opened with a right thoracotomy incision. Positive pressure respiration was begun immediately prior to entering the chest cavity. In the earliest experiments the respirator was a Phipps-Bird model 71-216 with a T cannula, the animal expiring directly through one short arm of the T to the external atmosphere. In later experiments a Phipps-Bird model 87801 was used, which had the advantage of utilizing a Y cannula, one arm of which was connected to a negative pressure port during the expiratory phase. In fact, there seemed to be no difference in ventilatory efficiency between these two models. There was, however, a marked difference noted in the myocardial high energy phosphates, depending upon the composition of the gas being supplied to the animal via the respirator. It will be shown in a later section that respiration with 100% oxygen leads to greater values of certain high energy phosphates under these operative conditions than does aritificial respiration with room air.

After beginning positive pressure respiration, the internal mammary arteries were clamped, the thoracotomy incision extended across the upper sternum, down the left axillary line, and finally across the lower thorax. This allowed the entire anterior chest wall to be removed in one piece and afforded excellent exposure of the heart. The heart and lungs were inspected carefully and if any operative trauma to these organs was evident the preparation was rejected. The pericardium was then carefully dissected free from the heart and a fine nylon suture was passed through the extreme apex of the heart. The amount of tissue transfixed by the needle was minute-- little more than a small amount of epicardium. The apex of the heart was lifted ventrally by very slight tension on this apical suture, the tracheal canula was pulled out at the height of an inspiration to permit rapid and complete collapse of the lungs away from the heart, and the heart was seized between two aluminum blocks attached to a pair of heavy forceps precooled in liquid nitrogen.

The aluminum-tipped forceps used were constructed after the description given by Wollenberger et al. (1960a). They consisted of two aluminum blocks, each measuring 4 x 5 x 1.25 cm, weighing 67.5 grams apiece, and were attached by screws to a metal clamp of the type used in automotive body repair shops (Hargrave spring clamp No. 3-- manufactured by the Cincinnati Tool Company). The clamp was self-closing by a powerful coil spring with a force of some 20 pounds weight. When the clamp was allowed to compress the heart in situ, the tissue was "instantly" frozen into plates of 1 to 2 millimeter thickness, and the blood was completely expressed from the ventricular cavities.

Wollenberger has shown (1960b) that under these conditions there is no loss of interstitial fluid.

Throughout the operation the temperature of the preparation was prevented from going below 34-35° by lights positioned above the dissecting board. An average of 30 to 35 minutes was required to obtain the heart, measured from the time of the injection of the anesthetic.

(2)

The frozen plates of myocardium were examined and all non-apical tissue and superficial blood vessels were scraped away with a scalpel cooled in liquid nitrogen. An estimated 300-400 mg of the cleaned apical muscle was placed in a small porcelain mortar precooled with liquid nitrogen and vigorously ground to a fine powder for 3 to 4 minutes with a porcelain pestal, also precooled in liquid nitrogen. All of the powder that could be conveniently scraped out of the mortar was rapidly transferred to a preweighed polypropylene test tube containing exactly two milliliters of ice cold 0.3N perchloric acid; the tube and contents were reweighed to obtain the weight of tissue taken for analysis. This suspension, kept in an ice bath, was immediately extracted with a high speed glass rod stirrer for three minutes. The contents of the tube were then centrifuged in the cold room for three minutes at 4000 x G. The clear supernatant was decanted into a glass centrifuge tube containing a measured quantity (about 0.18 ml) of ice cold 3N KOH and 0.1M versene in the ratio of 0.011 ml of versene per 100 mg of tissue. The solution was adjusted to pH 7 by the addition of small amounts of 3N KOH or 3N perchloric acid, as necessary--the quantities used being too small to effect the final volume of the extract. The pH was estimated with Hydrion paper

to an accuracy of ±0.1 pH unit. The neutral solution was allowed to stand in an ice bath for one hour to permit the precipitation of perchlorate salts, and the solution was once again centrifuged in the cold. This gave the final, neutral, extract containing the acid soluble phosphate compounds of the muscle, ready for chromatographic separation.

(3)

A paperchromatographic separation, modified from the original procedures of Fleckenstein and Janke (1953), Fleckenstein (1955), and Gerlach and Janke (1958), was used for the quantative determination of the phosphorus-containing compounds in the muscle extract. The paper employed, in all instances, was Whatman No. 1 chromatography grade, pretreated by washing with ethylenediaminetetraacetic acid disodium salt (5 gm per liter of aqueous solution, adjusted to pH 8.7 with concentrated NaOH) for 45 minutes. The papers were then washed six times with distilled water and allowed to dry overnight. This treatment appreciably improves the quality of the subsequent separation by removing many of the interfering heavy metals.

Four sheets of this paper, 20 x 45 cm, were used for each separate muscle extract (see fig. 3 for a chromatography flow sheet). Two of the four papers were used for the duplicate determination of CP and Pi; the other two were used for the determination of ATP, ADP, and AMP in duplicate. Each of the four papers received 100 microliters of the ice cold muscle extract, dispensed in twenty 5.00 microliter drops from a Lang-Levy type constriction pipette (Arthur H. Thomas Co.) with a guaranteed accuracy of ±0.5%. The spots were dried between applications by fans blowing room air.

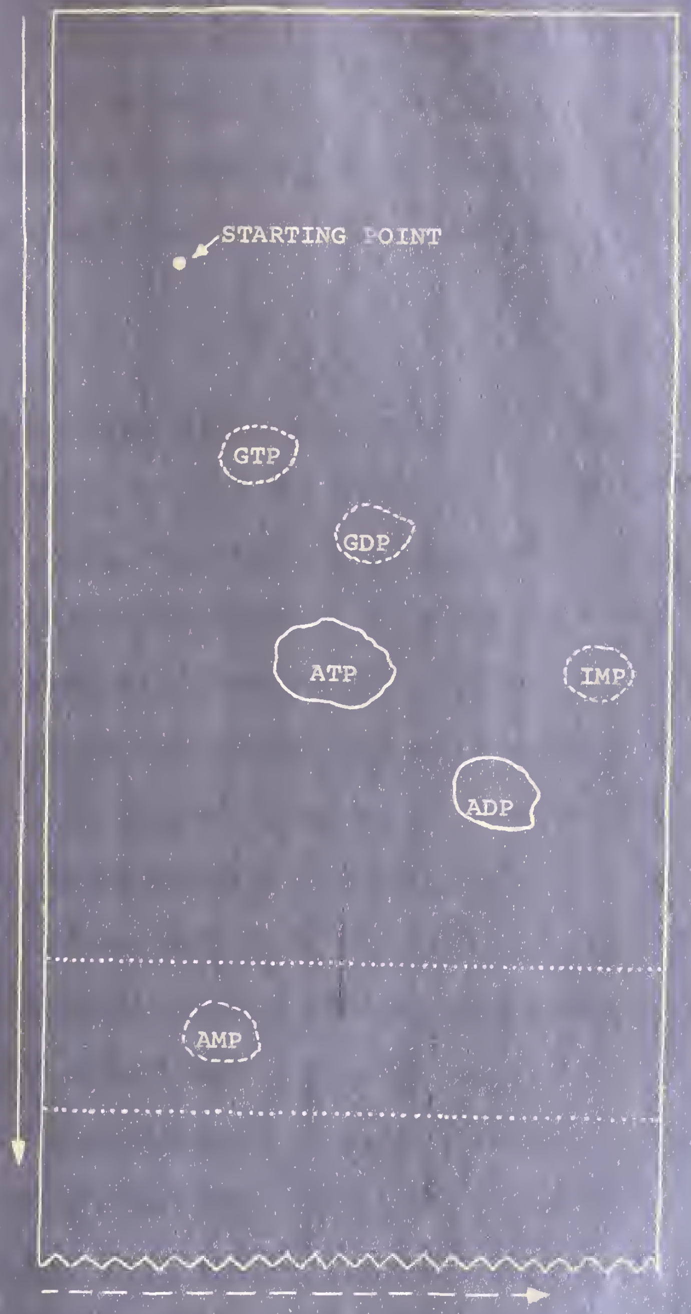

Fig. 1. The positions of the various UV-absorbing nucleotides on the paper chromatogram following two dimensional separation. (⟶) indicates first dimension. (- - - →) indicates second dimension. The dotted lines indicate the strip of AMP removed for second dimension purification in solvent D4. The AMP will be observed to undergo practically no migration in the second dimension. No versene spot is visible after the first dimension treatment with solvent IIb or IIbm.
(Approximately 1/2 size).

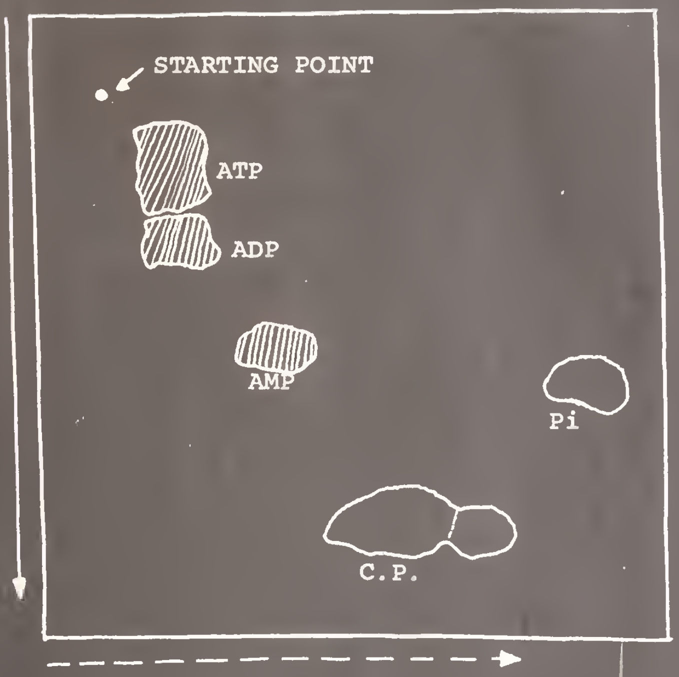

Fig. 2. The positions of the phosphate compounds of the myocardial muscle extract following two dimensional separation in solvent K.P. (first dimension) and D_4 (second dimension). (⟶) indicates first dimension; (----→) indicates second dimension. (Approximately 1/2 size).

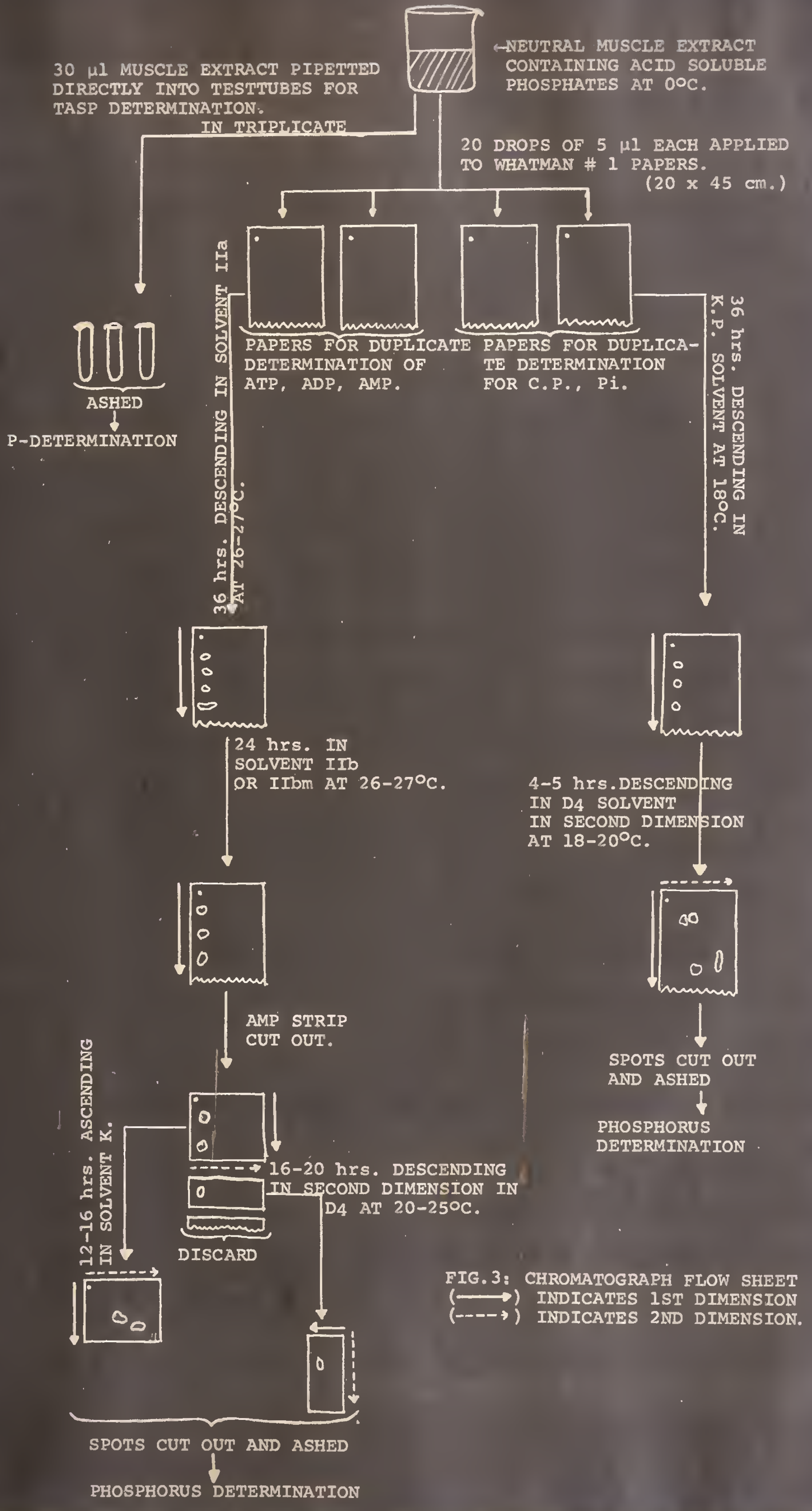
←NEUTRAL MUSCLE EXTRACT CONTAINING ACID SOLUBLE PHOSPHATES AT 0°C.
30 μl MUSCLE EXTRACT PIPETTED DIRECTLY INTO TESTTUBES FOR TASP DETERMINATION.
IN TRIPLICATE
20 DROPS OF 5 μl EACH APPLIED TO WHATMAN # 1 PAPERS. (20 x 45 cm.)
ASHED
P-DETERMINATION
PAPERS FOR DUPLICATE DETERMINATION OF ATP, ADP, AMP.
PAPERS FOR DUPLICATE DETERMINATION FOR C.P., Pi.
36 hrs. DESCENDING IN SOLVENT IIa AT 26-27°C.
36 hrs. DESCENDING IN K.P. SOLVENT AT 18°C.
24 hrs. IN SOLVENT IIb OR IIbm AT 26-27°C.
4-5 hrs. DESCENDING IN D4 SOLVENT IN SECOND DIMENSION AT 18-20°C.
AMP STRIP CUT OUT.
SPOTS CUT OUT AND ASHED
PHOSPHORUS DETERMINATION
12-16 hrs. ASCENDING IN SOLVENT K.
16-20 hrs. DESCENDING IN SECOND DIMENSION IN D4 AT 20-25°C.
DISCARD
SPOTS CUT OUT AND ASHED
PHOSPHORUS DETERMINATION

FIG.3: CHROMATOGRAPH FLOW SHEET
(——→) INDICATES 1ST DIMENSION
(- - - →) INDICATES 2ND DIMENSION.

The two papers used for the determination of the nucleotides were run descending for 36 hours in an alkaline solvent (IIa) at 26-27° (the composition of the individual chromatography solvents is given in Appendix I). The chromatography jar must be enclosed in a cabinet or barrel, preferably double walled, to protect the system from drafts. For the adequate separation of the nucleotides, in particular that of ATP from ADP, this temperature must be strictly adhered to. Should the ambient temperature be allowed to fall below 25° for any appreciable period, poor separation of the ATP and ADP will result. If the temperature exceeds 28° for more than a brief time, ATP decomposes with the production of ADP and AMP in appreciable amounts in this alkaline medium. The occurrence of decomposition can be detected by inspection of the chromatogram under short wave UV light, when a UV-absorbing "isthmus" can be seen connecting the ATP and ADP spots.

After 36 hours in solvent IIa, the nucleotide papers were removed from the chromatography jar and dried in room air. They were inspected under UV light of 260 millimicrons for adequacy of the initial separation. The light source used was constructed according to the protocol of Markham and Smith (1949). At this point there were (at least) four spots visible (fig. 1). Exactly at the starting point was a "ghost" spot of substances of unknown composition which absorb light of this frequency but which do not move in this solvent system. Next, were observed the faint outlines of GTP and GDP. Then came a large intensely absorbent spot--the most striking component of the chromatogram--which was ATP. Several centimeters further along the chromatogram was a fainter spot, ADP. About twice the distance from

ADP as the ADP was from the ATP, a very faint spot corresponding to AMP was found. Beyond AMP a long, wide "smudge", appearing greenish-yellow under the UV and having a "handle bar mustache" configuration, was usually prominent; this was versene.

If the compounds showed adequate separation at that point, the chromatograms were replaced in the chromatography chamber to run descending for an additional 24 hours in the first dimension in solvent IIb at the same temperature as for the IIa run. Should there have been a slow migration of the nucleotides in solvent IIa, a modification of IIb, known as IIbm, was used. Solvent IIbm allowed more rapid migration of all three nucleotides than did IIb but did not effect the relative positions of the compounds to each other.

After 24 hours in solvent IIb (or IIbm) the papers were once more removed from the jar, dried and inspected. The versene "smudge" was observed to have been washed out of the paper. The AMP spot was located under UV, and a horizontal strip of the chromatogram containing this compound was cut away (figs. 1 and 3). A leader of washed paper was sewn to the end of the chromatographic strip nearest to the AMP spot, and a trailer strip of unwashed paper was sewn to the other end. These AMP chromatograms were run descending in solvent D_4 in the second dimension at 20-25° for 16-18 hours. This special treatment of AMP was necessitated by the fact that a non-UV-absorbing, phosphorus-containing compound migrates together with the AMP in solvents IIa and IIb. Hochrein and Döring (1958) believe this to be a phosphotriose. In any event, if the AMP spot was analyzed for its phosphorus content after running in solvents IIa and IIb, values for AMP approaching those for ATP, on a molar basis, would sometimes be obtained. This was

impossible, as inspection of the chromatogram under UV disclosed only trace amounts of AMP. However, after 16 hours in solvents D_4 the AMP had hardly changed its position while the phosphorylated sugar has migrated well down the chromatogram, leaving pure AMP behind. After the AMP strip had been removed and dried, the AMP was located under the UV lamp, the spot circled in pencil, cut from the paper, and was ready for a phosphorous determination.

The remainder of the chromatogram, the upper portion containing the ADP and ATP, was run ascending at 20-25° in the second dimension, in solvent K for 16-18 hours. (This solvent must be allowed to stand for 24 hours at room temperature after mixing and prior to use). Following completion of this run, the papers were again dried and inspected under the UV light. The positions of the ATP and ADP spots were noted and the appropriate areas cut from the chromatogram for phosphorous analysis.

The two papers used for the determination of CP and Pi were run descending in the first dimension in solvent K.P. for 36 hours. Here again, temperature is of critical importance. The ideal temperature appeared to be 18-20° for this solvent system. Above this temperature there was some hydrolysis of the CP with a consequent increase in the Pi fraction; at lower temperatures the CP showed poor separation from the Pi. If it is impossible to obtain temperatures in the optimum range, a compromise can be made by running the system in the "warmest cold room" obtainable for periods approaching 50 hours.

Following the first dimension separation, the CP was found to have migrated appreciably further than the Pi, which in turn had progressed farther than AMP; the ATP and ADP remained practically

unseparated at the starting point.

In the second dimension these papers were run descending for 4 to 5 hours (not more) at room temperature in solvent D4. In this acid solvent there was some hydrolysis of the CP during this run. However, the Pi formed migrated directly in front of the unhydrolyzed CP and had the net effect of producing one elongated "double humped" spot and all of the phosphorus contained in the original CP could easily be recovered.

As neither CP nor Pi absorb UV light, their positions were fixed by spraying the chromatograms with the molybdate reagent of Hanes and Isherwood (1949), the composition of which is given in Appendix I. The reagent was sprayed on the chromatograms until they were thoroughly damp, and the papers were hung in an oven for seven minutes at 85° to effect hydrolysis of all the phosphate esters. Upon remoistening the chromatograms with water vapor from a steam kettle, and exposure to UV-light (sun lamp) or to a hydrogen sulphide atmosphere, the phosphorus-containing areas became visible as intense blue spots on a buff background. They were then circled and immediately cut from the chromatogramms, as delay could result in the spots fading or the entire paper gradually becoming blue, especially on exposure to sunlight.

Although there is theoretically no need to use the molybdate spray reagent on the chromatography papers used for the nucleotide determination, since these compounds are visible under the UV light, it was found that there was a practical advantage to doing this, in that the subsequent ashing of the paper spots for the phosphorus determination was accomplished much more quickly and smoothly. The combination of acid and heat in the "bluing" procedure effects a "predigestion" of

the paper.

(4)

The compounds to be determined were located on the chromatograms either by UV light or spraying with molybdate reagent as described in the previous section. They were then cut from the chromatogram in the shape of rectangles or squares, and the areas of the paper spots recorded. From each chromatogram a 10 cm^2 paper blank was cut from an area which lay behind the initial starting point. These phosphorus-containing spots and paper blanks were then wet ashed in Pyrex test tubes over a Kjeldahl burner stand, using an ashing reagent composed of two parts of 60% perchloric acid + three parts of concentrated sulphuric acid. The reagent was used in quantities roughly proportional to the area of the paper spot being ashed; for spots less than 10 cm^2, 0.5 ml of the reagent was used, for papers between 10 and 15 cm^2, 0.7 ml was preferable. Upon heating the paper with this reagent, the solution was observed to change from almost black to light orange, then through a yellow tint, and finally became colorless. On continued heating, the colorless solution would suddenly turn and remain yellow-green. The heating was continued for five minutes after this final change. At that point, all of the organic phosphates had been converted to the inorganic form.

For the determination of the TASP fraction of the muscle extract, 30 microliter aliquots of the neutral extract were pipetted directly into Pyrex test tubes with a constriction pipette, 0.7 ml of ashing reagent added, and the same heating procedure used as described above for the paper spots.

A modification of the method of Berenblum and Chain (1938) was used for the determination of phosphorus. In this procedure, phosphomolybdic acid is formed by the reaction of ammonium molybdate with the inorganic phosphate, and the phosphomolybdic acid is subsequently extracted into isobutanol where it is reduced to a blue compound with excess stannous chloride. The intensity of the color is determined photometrically. This was found to be an exceedingly sensitive, stable, and reproducable method for the determination of small amounts of phosphorus. Using a Klett filter photometer with a 660 millimicron filter it was possible to measure concentrations over the range 0.1 to 8+ micrograms of P in a final volume of 5 ml with an error of ±0.05 micrograms for a single determination. The complete experimental protocol as used in these experiments is given in Appendix II.

(5)

The photometer reading for a given compound must be corrected for both reagent and paper (chromatogram) blanks. This having been done, the quantity of phosphorus contained in the particular compound cut from the chromatogram can be obtained by reference to a standard curve. With appropriate factors for the weight of tissue taken for analysis and the extraction volume, the concentrations of the various acid soluble phosphates may be expressed both in terms of micromoles of compound per gram of fresh tissue and as a percentage of the TASP. Details of these calculations are given in Appendix III.

Animal number and Respirator gas	TASP		ATP		ADP		AMP		CP		Pi	
	µM/g	%TASP	µM/g	%TASP	µM/g	%TASP	µM/g	%TASP	µM/g	%TASP	µM/g	%TASP
A1 - air	34.2	100	2.80	24.6	0.59	3.4	0.92	2.70	5.67	16.6	3.00	8.75
A2 - air	38.5	100	3.29	25.6	0.81	4.2	1.10	2.90	5.03	13.1	2.87	7.45
A3 - air	33.1	100	2.98	27.0	0.58	2.6	1.86	5.64	3.32	10.0	3.89	11.80
A4 - air	34.8	100	2.96	26.2	1.05	6.0	0.78	2.20	5.00	14.4	4.61	13.20
A5 - oxygen	37.4	100	3.26	26.1	0.86	4.6	0.54	1.45	6.74	18.0	2.60	6.96
A6 - oxygen	36.8	100	3.46	28.2	0.82	4.5	0.67	2.00	7.67	20.9	3.32	9.02
A7 - oxygen	35.2	100	3.55	30.2	0.75	4.3	0.58	1.65	7.77	22.0	3.05	8.67
A8 - oxygen	36.4	100	3.42	28.2	0.72	4.0	0.62	1.70	6.96	19.1	2.98	8.16
A9 - oxygen	34.8	100	3.59	30.9	0.78	4.5	0.50	1.40	7.53	21.6	3.12	8.95
A10 - oxygen	35.9	100	3.63	30.2	0.74	4.2	0.39	1.10	7.03	19.6	3.58	10.00
A11 - oxygen	39.2	100	4.86	37.2	0.83	3.2	0.45	1.20	8.18	20.9	3.02	7.71
Mean of A5-A11	36.53	100	3.681	30.14	0.786*	4.19*	0.536	1.50	7.411	20.30	3.096*	8.496*
± S.E. of Mean	±0.56		±0.202	±1.33	±0.020	±0.18	±0.037	±0.12	±0.195	±0.55	±0.115	±0.374

*Differs significantly from hyperthyroid animals. See text for probability values.

Table 1. The steady state concentrations of the most important acid soluble phosphate compounds in the apical myocardium of the normal guinea pig. Anesthesia with pentobarbital, thoracotomy, and _in situ_ freezing of the heart between aluminum blocks, precooled in liquid nitrogen.

Animal number and Respirator gas	TASP		ATP		ADP		AMP		CP		Pi	
	μM/g	%TASP	μM/g	%TASP	μM/g	%TASP	μM/g	%TASP	μM/g	%TASP	μM/g	%TASP
T1 - air	32.0	100	3.14	29.5	0.80	5.0	2.56	8.02	3.09	9.68	-	-
T2 - air	35.7	100	4.34	36.4	0.75	4.2	0.78	2.19	6.56	18.40	2.62	7.34
T3 - oxygen	36.1	100	3.68	30.6	1.03	5.7	0.63	1.70	6.56	18.20	3.63	10.10
T4 - oxygen	34.7	100	3.98	34.2	0.84	4.8	0.71	2.00	6.35	18.30	3.75	10.80
T5 - oxygen	36.1	100	3.71	30.8	0.79	4.4	0.64	1.80	6.73	18.60	3.42	9.46
T6 - oxygen	35.0	100	3.57	30.6	0.85	4.9	0.55	1.60	6.94	19.80	3.51	10.00
T7 - oxygen	37.8	100	4.10	30.8	0.89	4.7	0.61	1.60	6.87	18.20	3.71	9.80
T8 - oxygen	35.6	100	3.63	30.6	0.82	4.6	0.52	1.50	7.26	20.40	3.55	9.94
T9 - oxygen	34.9	100	3.38	29.0	0.84	4.8	0.54	1.50	7.53	21.60	3.62	10.40
Mean of T3-T9	35.74	100	3.721	30.94	0.866*	4.84*	0.600	1.67	6.891	19.30	3.599*	10.07*
± S.E. of Mean	±0.40		±0.093	±0.59	±0.030	±0.16	±0.025	±0.07	±0.152	±0.50	±0.043	±0.16

*Differs significantly from control animals. See text for probability values.

Table 2. Steady state concentrations of the most important acid soluble phosphate compounds in the apical myocardium of thyrotoxic guinea pigs. (Daily injections of 80-100 μg of thyroxine per kilogram body weight for 14-18 days). Pentobarbital anesthesia, thoracotomy, and in situ freezing of the heart between aluminum blocks, precooled in liquid nitrogen.

	CONCENTRATION IN μM/GM. OF FRESH TISSUE					
TISSUE ANALYZED	ATP	ADP	AMP	Pi	CP	REFERENCE
Guinea pig left ventricle *in situ*	3.48	1.02	-	1.66	7.20	Hochrein and Döring, 1958
Guinea pig left ventricle *in situ*	5.59	0.64	0.34	3.71	8.38	Feinstein, 1960
Guinea pig left ventricle in HLP	3.15	0.88	-	2.78	7.01	Hochrein and Döring, 1958
Guinea pig apical myocardium *in situ*	3.68	0.79	0.54	3.10	7.41	Brenckman and Goodkind, 1963
Guinea pig left atrium *in vitro*, 30°	2.41	0.43	0.23	6.24	4.19	Furchgott, de Gubareff, 1958
Guinea pig left ventricle *in situ*	-	-	-	2.0	10.9	Wollenberger et al., 1960b
Rabbit left ventricle *in situ*	-	-	-	1.9	8.2	Wollenberger et al., 1960b
Rabbit apical myocardium *in situ*	-	-	-	4.10	5.43	Fawaz and Manoukian, 1962
Rabbit atria *in vitro*, 30°	2.21	0.28	0.31	4.3	3.9	Fleckenstein et al., 1959
Rat ventricle *in situ*	4.12	0.71	0.53	3.8	6.8	Fleckenstein et al., 1959
Rat ventricle *in situ*	-	-	-	5.23	5.58	Fawaz and Manoukian, 1962
Cat apical myocardium *in situ*	3.39	0.69	0.19	5.7*	5.2*	Fleckenstein et al., 1959
Cat papillary muscle *in vitro*	3.81	0.22	0.17	3.2	7.04	Lee et al., 1961
Dog left ventricle (apex) *in situ*	5.7	-	-	1.9	12.8	Wollenberger et al., 1960b
Dog left ventricle *in situ*	-	-	-	4.84	7.81	Fawaz and Manoukian, 1962

*Indicates that some conversion of CP to Pi is believed to have taken place during the freezing procedure.

Table 3. Experimental values for the steady state concentrations of the adenine nucleotides, creatine phosphate, and inorganic phosphate in various cardiac muscle preparations. (Taken in part from table 1 of Furchgott and Lee, 1961).

EXPERIMENTAL RESULTS

In Table 1 are listed the micromolar concentrations of the TASP, ATP, ADP, AMP, CP, and Pi fractions as found in heart muscle from eleven normal guinea pigs; also listed are the percentages of the TASP-P which the individual compounds accounted for. Table 2 summarizes the same data for the nine hyperthyroid animals. For comparison with Table 1, values from the recent literature for the acid soluble phosphate composition of mammalian myocardium are included in Table 3.

EFFECT OF GAS MIXTURE FOR RESPIRATION. The first four animals in the control series (A1-A4) and the first two hyperthyroid animals (T1 and T2) were allowed to breath room air through the respirator, while subsequent animals (A5-A11 and T3-T9) were given 100% oxygen during the period of artificial respiration. The substitution of oxygen for air had a pronounced effect upon the concentrations of both the adenine nucleotides and the CP and Pi. In the control animals there was a clear increase in the mean ATP concentration from 3.01 μM/gm for animals A1-A4, to 3.68 μM/gm for A5-A11. Though the ADP concentrations were apparently unaffected by the change in gas mixtures, levels of AMP were markedly higher in the animals breathing air as opposed to those receiving oxygen (1.16 μM/gm vs. 0.54 μM/gm). Even more striking were the effects produced in the CP and Pi fractions upon changing from air to oxygen; the mean CP concentration of animals A1-A4 was only 4.76 μM/gm (13.52% of the TASP), whereas for animals A5-A11 there was 7.41 μM/gm (20.3% of the TASP). Concomitant with the rise in the CP concentration during respiration

with oxygen, there was a decrease in the mean Pi levels from 3.59 μM/gm (10.30% of TASP) in animals A1-A4 to 3.10 μM/gm (8.50% of TASP) for animals A5-A11. This was reflected in the CP:Pi ratios for two groups; CP:Pi for A1-A4 was 1.39, in contrast to 2.41 ± 0.09 for A5-A11. There were two hyperthyroid animals (T1 and T2) that were given air to breath; animal T1 shows all of the characteristics enumerated above, viz. low ATP, markedly elevated AMP, and a grossly low CP, when compared with animals T3-T9. Surprisingly, animal T2 seems to have faired rather well breathing room air and, indeed, had the highest ATP value recorded in the hyperthyroid series, while its CP was normal, and its Pi somewhat lower than the mean.

These findings strongly suggest that, except in the case of T2, artificial respiration with air under the operative conditions employed was insufficient to prevent a rather pronounced degree of anoxemia. All the hallmarks of anoxia were present in these animals--a moderate decrease in ATP concentration, with a severe depression of the CP fraction and rise in the Pi levels. For this reason, further discussion of experimental results will be confined to those animals where this condition was not believed to have obtained, i.e. A5-A11 and T3-T9.

THE TOTAL ACID SOLUBLE PHOSPHATE FRACTION. In the present series of experiments the TASP fraction of the myocardium was found to be 36.53 ± 0.56 μM/gm in the control animals, and 35.74 ± 0.40 μM/gm for the hyperthyroid animals. The difference between these mean values, determined from the Student distribution*, was not

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*"P" was obtained from the table of "Significance Limits of the Student Distribution"; Documenta Geigy, Scientific Tables, 6th edition, p. 32, Basel, 1963.

statistically significant, P being between 0.20 and 0.30. (In all subsequent discussion, we shall consider a P value of less than 0.05 as "significant", while a value of less than 0.005 is "highly significant"). It is interesting to observe that the mean TASP value of the four anoxic control animals, 35.15μM/gm, does not differ appreciably from the value for animals A5-A11.

Values for TASP are not included in Table 3, but in Hochrein and Döring's (1958) in situ guinea pig left ventricle preparation a value of 31.20μM/gm was noted, while in their guinea pig left ventricle in the heart-lung preparation (1958) a value of 29.39μM/gm was found. Wollenberger et al. (1960b) observed a larger value of 47.6 ± 8.6μM/gm for his in situ guinea pig apical tissue, obtained by freezing between aluminum blocks precooled in liquid nitrogen. They also found that the mean TASP concentration in the myocardium of three guinea pigs whose hearts were excised and immediately immersed in liquid nitrogen was only 35.5 ± 2.6μM/gm. It was concluded that removal of the heart between the aluminum blocks prevents a certain amount of TASP "loss". In view of the fact that our animals, all of whose hearts were obtained with the aluminum block technique, had a value appreciably lower than Wollenberger's equivalent preparations, the question of a difference in animal strains becomes a real one.

THE ATP FRACTION. The mean concentration of ATP in the control animals (A5-A11) was 3.68 ± 0.20μM/gm of tissue (= 342 ± 19 micrograms of P/gm = 30.1 ± 1.3% of the TASP; for the hyperthyroid animals (T3-T9) the corresponding values were 3.72 ± 0.09μM/gm of tissue (= 346 ± 9 micrograms of P/gm = 30.94 ± 0.59% of the TASP). Neither the difference between the mean micromolar concentrations ($P>0.8<0.9$)

nor the percentage of the TASP ($P>0.5<0.6$) was significant.

These control levels of ATP are in agreement with those obtained by Hochrein and Döring (1958) who utilized the same experimental procedures as we did, while they are somewhat lower than those found by Feinstein (1960) who used a sensitive enzymatic method. As Hochrein and Döring's value of 3.48 μM/gm was obtained in animals having a TASP of 31.20 μM/gm, it can be calculated that their ATP-P accounted for 33.5% of the TASP--a value not very different from that obtained in our animals.

THE ADP FRACTION. The mean value of the myocardial ADP in the present series of animals was 0.79 ± 0.02 μM/gm (= 48.9 ± 1.2 micrograms of P/gm = 4.2 ± 0.2% of the TASP) for the control animals, and 0.87 ± 0.03 μM/gm (= 53.7 ± 1.8 micrograms of P/gm = 4.8 ± 0.2% of the TASP) for the animals receiving thyroxine. The difference between the mean micromolar ADP concentrations in the two series is of borderline significance, $P = 0.05$, while the difference between the mean TASP percentages has a P value between 0.01 and 0.02.

Reference to Table 3 will show that the control ADP concentration is in the same range as those reported for similar preparations, while our value of 4.2% of the TASP-P is slightly lower than Hochrein and Döring's (1958) figure of 6.55% based on an ADP concentration of 1.20 μM/gm and a TASP of 31.20 μM/gm.

THE AMP FRACTION. The mean myocardial AMP concentration of animals A5-A9 was 0.54 ± 0.04 μM/gm (= 16.5 ± 1.2 micrograms of P/gm = 1.50 ± 0.12% of the TASP), and for the hyperthyroid animals (T3-T9) 0.60 ± 0.03 μM/gm (= 18.6 ± 0.8 micrograms of P/gm = 1.67 ± 0.07% of the TASP). The difference between these values is not statistically

significant either for the micromolar concentrations ($P > 0.10 < 0.20$), or for the TASP percentages ($P > 0.20 < 0.30$).

There is only one value reported in the literature for guinea pig myocardial AMP concentration, that of Feinstein (1960), which is given as 0.34 µM/gm of tissue. As no value for TASP is mentioned in his paper, it is impossible to say what percentage of the TASP was accounted for by AMP.

THE CREATINE PHOSPHATE FRACTION. The mean micromolar CP concentration in the well-oxygenated control animals was found to be 7.41 ± 0.20 µM/gm (= 230 ± 6 micrograms of P/gm = 20.30 ± 0.55% of the TASP). The hyperthyroid animals had a mean CP concentration of 6.89 ± 0.15 µM/gm (= 214 ± 5 micrograms of P/gm = 19.30 ± 0.50% of the TASP).

In Table 3 are listed some recent values for CP in various guinea pig preparations. In Hochrein and Döring's (1958) in situ guinea pig preparations, CP was found to constitute 23.04 ± 0.89% of the TASP, while in Wollenberger et al.'s (1960b) in situ guinea pig ventricles, 22.8% of the TASP was contained in the CP fraction. The significance of our values for CP will be discussed in greater detail in the final section.

THE INORGANIC PHOSPHATE FRACTION. In the control group (A5-A11) the micromolar concentration of Pi was determined as 3.10 ± 0.12 µM/gm (= 95.9 ± 3.4 micrograms of P/gm = 8.50 ± 0.37% of the TASP). The hyperthyroid animals' hearts had a mean Pi concentration of 3.60 ± 0.04 µM/gm (= 111.6 ± 1.3 micrograms of P/gm = 10.07 ± 0.16% of the TASP). The difference between the mean values of both the micromolar concentrations and the TASP percentages are highly significant, P

being between 0.001 and 0.005 in each instance. Reference to Table 3 shows that the reported values for Pi in guinea pig myocardium have a rather wide range, our present values being less than the figure of 3.71 μM/gm reported by Feinstein (1960), but greater than Hochrein and Döring's (1958) value of 1.66 μM/gm. The latter authors found that Pi accounted for 5.33% of their TASP, while Wollenberger et al.'s (1960b) value of 2.0 μM/gm constituted 4.2% of the TASP.

RECOVERY PERCENTAGES. Summation of the ATP, ADP, AMP, CP, and Pi fractions of the well-oxygenated euthyroid animals shows a mean micromolar concentration of 15.51 ± 0.39 μM/gm (= 735 ± 24 micrograms of P/gm = 64.63 ± 1.62% of the TASP); i.e. in the control animals 64.63% of the TASP could be accounted for by these five compounds. The hyperthyroid animals had a micromolar sum of 15.68 ± 0.12 μM/gm (= 744 ± 8 micrograms of P/gm = 66.83 ± 0.64% of the TASP). The difference between the recovery percentages of the five compounds in the two series is not statistically significant, ($P>0.20<0.30$). In the control animals about half of the TASP (50.4 ± 1.8%) was contained in the ATP and CP fractions. This figure is almost identical for the hyperthyroid series (50.2 ± 1.1%). The comparable figure for Hochrein and Döring's (1958) *in situ* guinea pig preparations is 56.5 ± 1.6%.

WATER CONTENT OF THE FRESH MYOCARDIAL TISSUE. In order to exclude the possibility that changes in the high energy phosphates were being caused, or concealed, by simultaneous alteration in the water content of the myocardium with the production of the hyperthyroid state, assays were performed on six normal guinea pigs and eight animals injected with 100 micrograms of thyroxine per kilogram of body weight for 14 days. The water content of the ventricular muscle, as determined by being between 0.001 [illegible] in each instance. Comparison [illegible] shows that the reported values for Pi in granite [illegible] rather wide range, [illegible] values [illegible] of 3.7[illegible] [illegible] (1960), [illegible] and Östing[illegible] (1959) [illegible] Pi accor[illegible] [illegible] (1960) [illegible] [illegible] Problems of [illegible] attenuation [illegible] [illegible] loss in tissue weight upon drying for 18 hours at 105°, was found to be 80.35 ± 0.30% (range, 79.8 - 81.8%) for the six control animals, and 80.04 ± 0.09% (range, 79.6 - 80.4%) for the hyperthyroid animals. The difference between the mean values is not significant, ($P>0.20<0.30$).

SUMMARY OF THE EXPERIMENTAL DATA. The injection of 80-100 micrograms of thyroxine per kilogram of body weight for periods of 14 to 18 days failed to produce any significant changes in the steady state concentrations of either the TASP, AMP, or CP fractions. An observed increase in the micromolar concentration of ADP in the hyperthyroid animals is of questionable significance. A highly significant increase in both the absolute level of Pi as well as the percentage of Pi in the TASP fraction was observed. The water content of the myocardium of the hyperthyroid animals did not differ from that of control animals. Careful inspection of the original data failed to disclose any correlation between either the weights of the animals, both euthyroid and hyperthyroid, and the concentrations of the high energy phosphates. Likewise, there was no correlation between the levels of these compounds found, and the small variations in thyroxine dosage (80 - 100 micrograms) or duration of treatment (14 - 18 days).

DISCUSSION

The concentrations of the various acid soluble phosphate compounds observed in the myocardium of our control animals agree well with the most recently reported values in the literature. At the outset of these experiments, the low values of ATP and CP obtained, together with high values of Pi, raised the suspicion of anoxia, an assumption which was later borne out by the reversal of these conditions with the use of 100% oxygen in the respirator. It was also expected that higher levels of CP might be obtained than those found by Hochrein and Döring (1958), since we were using the technique of in situ freezing between aluminum blocks precooled in liquid nitrogen, whereas the former workers employed simple excision of the operatively exposed heart with subsequent plunging into liquid nitrogen. In Wollenberger et al.'s (1960b) experience, use of the aluminum block procedure afforded mean values of 10.9 μM/gm for CP, as opposed to a mean level of 7.0 μM/gm with simple excision of the heart and immediate immersion in liquid nitrogen. In fact, values not significantly higher than those of Hochrein and Döring were obtained, while the high levels found by Wollenberger's group were never observed.

While these experiments were in progress, a report appeared (Fawaz and Manoukian, 1962) in which the Wollenberger technique was compared with simple excision and freezing in liquid nitrogen; these authors found that the aluminum block method of freezing led to slightly higher levels of CP in dog and rabbit hearts, but yielded appreciably higher values in rat hearts. The reason for this difference has not been elucidated. As our experiments did not include any animals whose hearts were obtained by the excision-immersion

technique, it is impossible to say whether or not lower values would have been obtained with such a procedure. Michal and Lamprecht (1961) have questioned Wollenberger's high CP values, feeling that he failed to completely extract the Pi from his preparations and, as CP was determined as the difference between Pi and the sum Pi+CP, a high apparent value for CP was the result.

Having no reason to doubt that a representative analysis of the acid soluble phosphate composition of the apical myocardium of normal guinea pigs was obtained, it remains to consider the differences observed in the hyperthyroid animals. As was shown in the section on Experimental Results, no statistically significant differences were noted in the steady state concentrations of the TASP, ATP, AMP, or CP in the hyperthyroid animals, as compared with the euthyroid controls. There was an ADP increase, both on a micromolar concentration and a percentage TASP-P basis, of borderline ($P>0.01<0.05$) significance, and a highly significant ($P>0.001<0.005$) increase in Pi concentration in the hyperthyroid animals.

The slight increase in the steady state level of ADP in the hyperthyroid animals is of particular interest in view of the central role in the regulation of oxidative metabolism which is now accorded this compound. Recent studies by Chance and Hess (1959a, 1959b, 1959c) have shown that the rate of oxidation by mitochondria is markedly increased by the addition of ADP, which acts as an acceptor of newly formed phosphate groups in the synthesis of ATP. Exhaustion of ADP results in a low rate of oxidative metabolism (1959a). In a system which has not undergone complete "uncoupling" of oxidative

phosphorylation (and hence would suffer a complete dissociation of oxygen consumption from ADP concentration) an increased concentration of ADP can act as a stimulus to the oxidative rate (Hoch, 1962).

In determining whether or not such a model can be applied to our hyperthyroid animals, several points must be taken into consideration. First, the minute oxygen consumption of the animals used in these experiments was not measured. It may be safely assumed, however, that the oxygen consumption was increased, in view of the relatively large doses of thyroxine administered and the significant weight losses observed (average of -12% after 11 days). Also, it has been shown that doses of thyroxine of the same magnitude as were employed in these animals cause a striking elevation in the guinea pig protein bound iodine levels. (Mean PBI level of euthyroid animals = 1.3 micrograms percent, range 0.7 - 1.8 μgm%; mean PBI of five animals receiving 100 micrograms of thyroxine per kilogram of body weight for 3+ weeks = 14.8 micrograms percent, range 8.6 - 20.7 μgm%)*.

This raises the second point, viz. that the doses of thyroxine used, while large, were not lethal. Several animals were continued on the usual dosage schedule for periods of four weeks without other effects than restlessness, failure to gain weight at the expected rate, and a poor coat of fur. In short, the animals were hyperthyroid, but not moribund. It is doubtful whether oxidative phosphorylation could be severely "uncoupled"--a potentially lethal situation--in animals capable of such a long period of survival.

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* Unpublished data, kindly furnished by Dr. M. Jay Goodkind.

With "normal" or "complete" coupling of oxidative phosphorylation the feed-back stimulation of oxidative metabolism by ADP serves as a regulatory mechanism for the cellular synthesis of ATP. If an "uncoupling" agent is present in sufficient concentration to be toxic, there is no possibility for rephosphorylation of ADP, and the further disintegration of ADP to AMP and IMP is likely. It is therefore of interest that Gerlach and Lübben (1958) observed that the decrease in ATP in pigeon erythrocytes incubated _in vitro_ with thyroxine (1 x 10^{-4} M) proceeded only to the ADP stage, whereas incubation with DNP resulted in the ATP being broken down to AMP and IMP.

The concept of "loose" coupling was introduced for the situation where oxidative metabolism proceeds at a high rate, at normal P:O ratios, but where the increased oxidative rate is independent of physiological changes in the ADP concentration. The possibility that an increase in ADP concentration occurred in our hyperthyroid animals is slight ($P=0.05$), and whether or not such an increase would have increased oxidative metabolism, as in "normal" coupling, or would have been without effect, as in "loose" coupling, is not known.

The increased level of myocardial Pi in the hyperthyroid animals is difficult to explain. It would be tempting to attribute the increased concentration of this compound to an equivalent decrease in the CP fraction, as the hyperthyroid animals had a mean CP concentration which was 0.52 μM/gm lower than the controls, while the Pi concentration was 0.50 μM/gm higher. The sum of the CP and Pi fractions, on a micromolar basis, was identical for the two groups--

10.51 ± 0.24 μM/gm for the control animals, and 10.49 ± 0.15 μM/gm for the hyperthyroid animals. It is impossible to definitely rule this possibility in or out, since the difference between the mean CP levels in the two groups is not statistically significant. It should be noted, however, that creatine phosphate, being the most labile compound isolated, being the most likely to suffer some degradation during the isolation procedure, and being present in relatively large amounts, could decompose sufficiently to supply a quantity of Pi that would cause an apparently significant increase in the Pi fraction without producing significant changes in the CP concentrations. (Similarly, a significant alteration in the concentration of any of the acid soluble phosphates which constitute only a small percentage of the TASP could easily fail to be reflected in the latter value.)

One further possibility for an increase in the Pi fraction may be considered, if only to supply a victim for Occam's razor. It is possible that under conditions which create an acceleration in high energy phosphate turnover, some other labile phosphorus compound, "XP", part of the 30+% of the TASP which was not accounted for, is utilized for phosphate transfer. The evidence for the existance of such a compound has been considered in the case of a muscle undergoing a single, brief twitch (see Introduction). The role and, particularly, the kinetics of phosphate transfer through such a compound in the chronic hyperthyroid state are difficult to formulate.

The point is eventually reached in which data derived from steady state concentration measurements must be supplemented by information regarding the turnover rates of these various high energy phosphate bonds. The energy which is made available to the muscle for the

performance of mechanical work is a function of both the steady state concentration of the energy rich compounds as well as the turnover rates of their high energy linkages. A valuable tool for investigating the nature of these dynamic changes is radiophosphorus in the form of $P^{32}O_4$. Thus, Fleckenstein et al. (1959) have shown that, while the quadriceps muscle of the rat has a much higher concentration of ATP (twice that of rat myocardium) and CP (three times that of rat myocardium), the rat myocardium incorporates $P^{32}O_4$ twenty times more rapidly into these compounds than does the skeletal muscle.

For investigating acute changes in in vitro preparations the uptake and incorporation of P^{32} serves as an excellent marker. For in vivo preparations, however, the radioactive marker is less informative because of the wide fluctuations in plasma activity among animals given identical dosages (A. Fleckenstein, personal communication). Even so, the relative rates of incorporation of P^{32} into the various phosphate groups, calculated as percentages of the plasma P^{32} activity, is extremely useful. In the present series of experiments the results of analyses of the steady state concentrations of the high energy phosphate compounds reveal a pattern which makes it extremely unlikely that oxidative phosphorylation is being "uncoupled" in our hyperthyroid animals. The very minimal differences that have been observed between the hyperthyroid and euthyroid animals naturally raise the question of whether or not the turnover rates of these compounds have been increased--an effect consistent with the concept of "loose coupling". In particular, knowledge of the rate of P^{32} incorporation into the CP and Pi fractions would

help to illuminate the origin of the observed Pi increase in the hyperthyroid animals--the genesis of which must otherwise remain open to speculation.

help to illustrate the vigour of the observed increase in the hyperthyroid animals--the genesis of which are [illegible] open to speculation.

SUMMARY AND CONCLUSIONS

Apical myocardium of animals rendered hyperthyroid by the daily injection of non-lethal doses of thyroxine showed only minimal changes in the concentrations of various high energy phosphate compounds, when compared with euthyroid controls. The concentrations of ATP, AMP, CP, and TASP were similar in the two groups. The ADP concentration in the hyperthyroid animals showed a slight increase in borderline significance ($P = 0.05$), while levels of Pi were significantly higher ($P > 0.001 < 0.005$) than those in the control animals. A likely explanation for the increase in Pi is an insignificant breakdown of CP in the hyperthyroid animals.

The slight differences observed in the steady state levels of these compounds in the two groups makes it extremely unlikely that "uncoupling" of oxidative phosphorylation occurred in the hyperthyroid animals. It is possible that "normal" or "complete" coupling was present--i.e. that a P:O ratio of 3 existed, with an increase in ADP concentration tending to increase the oxidative rate. One would not necessarily expect to find increased concentrations of ATP in such circumstances if the turnover rates of the high energy bonds were also increased. The phenomenon of "loose" coupling implies an increase in the rate of oxidative metabolism, with normal P:O ratios, but without the need of increased ADP concentration for stimulation of the oxidative rate. This situation might also have been present in the hyperthyroid animals. Measurement of mitochondrial P:O ratios, phosphorus turnover rates, and oxidative rates at several ADP concentrations would help to decide between these last two possibilities.

The technique used to obtain the muscle tissue for high energy phosphate analysis is of the utmost importance if breakdown of ATP and CP is to be prevented during the isolation procedure. Adequate oxygenation of the preparation during operative removal or *in situ* freezing of the tissue is absolutely necessary. The animals receiving 100% oxygen through the respirator had higher CP and ATP levels, and lower Pi levels, than animals receiving room air. It is felt that the action of pure oxygen in these preparations was to counteract anoxia secondary to the accumulation of mucous secretions in the respiratory tract during anesthesia.

Rapid freezing of the myocardium is also necessary if breakdown of CP is to be avoided. The Wollenberger technique of *in situ* freezing between aluminum blocks precooled in liquid nitrogen was used in all these experiments and was found to be simply and satisfactory.

APPENDIX I

Composition of reagents and solvents for chromatography.

SOLVENT IIa

8-oxyquinoline.....5 mg
ethanol............5 ml
n-propanol........25 ml
n-butanol.........30 ml
ammonia (25%).....30 ml
distilled water...10 ml

SOLVENT K

trichloro-
acetic acid (30%)...15 ml
formic acid (96%)...20 ml
n-butanol...........40 ml
n-propanol..........20 ml
acetone.............25 ml
distilled water......5 ml

MOLYBDATE SPRAY REAGENT

3 grams of ammonium molybdate dissolved in 150 ml of distilled water.

15 ml of 60% perchloric acid

4.38 ml of 25% hydrochloric acid,

distilled water to make 300 ml of solution.

SOLVENT IIb

Same composition as solvent IIa, except that 8-oxyquinoline is omitted.

SOLVENT IIbm

ethanol............5 ml
n-propanol........30 ml
n-butanol.........25 ml
ammonia (25%).....30 ml
distilled water...10 ml

SOLVENT D_4

diisopropyl ether....40 ml
n-butanol............30 ml
formic acid (96%)....23 ml

SOLVENT K.P.

methanol..........45 ml
isopropanol.......30 ml
ammonia (25%).....15 ml
distilled water...10 ml

APPENDIX II

THE COLORIMETRIC DETERMINATION OF PHOSPHORUS.*

1. Wash contents of ashing tube into a clean 30 ml separatory funnel with 7 ml of distilled water, divided into three portions.
2. Rinse ashing tube with 2.5 ml of 5% (W/V) aqueous ammonium molybdate and add to separatory funnel.
3. Add 5.00 ml of isobutanol to the contents of the separatory funnel with a volumetric pipette.
4. Shake vigorously for 60 seconds.
5. Allow layers to separate; draw off the discard the lower (aqueous) layer.
6. Add 5 ml of 1N sulphuric acid.
7. Shake for 15 seconds, allow layers to separate, draw off and discard the lower (acid) layer.
8. Repeat steps 6 and 7 once more.
9. Run the isobutanol into a clean Klett tube having a graduation mark at 5 ml.
10. Rinse the separatory funnel into the Klett tube with approximately 1 ml of 95% ethanol.
11. Add 0.1 ml of stannous chloride solution (2 grams of stannous chloride dissolved in concentrated hydrochloric acid to give a final volume of 5 ml).
12. Add sufficient 95% ethanol to bring the contents of the Klett tube to the 5 ml graduation.

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* Modification of the procedure of Berenblum and Chain (1938).

13. Invert the Klett tubes several times to bring about complete dispersion of the reagents.

14. After five minutes, read in a Klett photometer with a 660 millimicron filter using pure isobutanol as a blank.

***

COMMENT. With the use of chemicals of the highest quality commercially available, but without further purifications, the reagent blank was quite low in these experiments (usually equivalent to 0.04 to 0.09 micrograms of phosphorus in the final 5 ml dilution).

For a given photometer and filter, a standard curve could be obtained that was reproducible for an indefinite time. This standard curve was plotted from values obtained by the analysis of 10 to 20 different amounts of phosphorus supplied by various volumes of a stock solution of dilute aqueous potassium dihydrogen phosphate (KH_2PO_4). Each time that a set of unknowns was to be analyzed a fresh stock solution of KH_2PO_4 was prepared and two or three points checked against the original standard curve. The results were invariably in agreement. If a new photometer or filter had to be used, as was necessary on several occasions, a new standard curve was determined. Actually, the differences between the machines and filters were insignificant.

Occasionally, when there was a long interval (weeks or months) between phosphorus determinations, abnormally high reagent values would be obtained. These would sometimes be 10 to 20 times higher than usual. It was found that this was due to the accumulation of a film on the inside of the Klett tube used for the phosphate

determinations, and proved resistant to removal with all organic solvents, mineral acids, and alkalies. Replacement with a new Klett tube showed that the reagent blanks actually had not changed. The accumulation of this film was most easily avoided by filling the tube with isobutanol during storage.

APPENDIX III

CALCULATION OF THE ACID SOLUBLE PHOSPHATE CONCENTRATIONS.

Calculation of the quantities of the acid soluble phosphate compounds present in the original tissue was done as follows. The Klett readings of the reagent blank, of the phosphorus containing compound to be determined, and of the appropriate paper blank were all converted into units of optical density (O.D.) by multiplication by a factor of 1/500. The O.D. of the reagent blank was then subtracted from the values of the phosphorus-containing spot and the paper blank. The standard curve was then consulted to find the value of P in micrograms corresponding to these optical densities. Since the P-value of the paper blank was determined for a paper of 10 cm^2, division of the P-value by 10 gives the quantity of "background" P per square centimeter of the chromatography paper. (This value was to some extent dependent upon the solvent systems in which the chromatogram had run. For the nucleotide papers treated with solvents IIa, IIb and K, the paper-P was usually in the range 0.01 - 0.02 micrograms of P/cm^2. For the papers treated with solvents K.P. and D_4, it usually lay between 0.008 and 0.015 micrograms of P/cm^2). The value of the paper-P/cm^2 was multiplied by the area of the paper spot ashed, and this product was subtracted from the total P-value given by the paper spot containing the compound. This is the number of micrograms of phosphorus in the given compound applied to the chromatogram, and is designated by n. Note that there is no correction for a paper blank in the determination of n in the case of the TASP fraction.

Having obtained n it remains to convert this value to micrograms (or micromoles) of phosphorus per gram of fresh tissue. Assume that A grams of myocardium were taken for analysis, that 2.000 ml of 0.3N perchloric acid were used for the extraction, that 0.180 ml of 3N KOH were used for neutralization, and that 0.030 ml of versene was added to the extract. Then, assuming the myocardium to have been 80.0% water, the total volume of fluid throughout which the acid soluble phosphate compounds could distribute themselves was:

$$0.800A + 2.000 + 0.180 + 0.030 = V \text{ ml}$$

However, only 100 microliters of this volume was applied to the chromatogram. Therefore, in the original extract there must have been

$$n \times \frac{V}{0.100}$$

micrograms of P contributed by the compound under consideration. Again, however, this amount of phosphorus was contained not in one gram of tissue, but only in A grams. To obtain the micrograms of phosphorus in one gram of tissue this expression must be multiplied by 1/A, or:

$$\text{micrograms of P in the compound} = n \times \frac{V \times 1}{0.100 \times A} \text{ per gram.}$$

For the TASP fraction, as only 0.030 ml of the extract was ashed and analyzed, this expression becomes:

$$\text{micrograms of acid soluble P} = n \times \frac{V \times 1}{0.030 \times A} \text{ per gram.}$$

To calculate the number of micromoles of a compound per gram of tissue, the number of micrograms is divided by 31 (the atomic weight of phosphorus) in the cases of the TASP, AMP, CP, and Pi. Since ADP

contains two atoms of P per molecule, the number of micrograms of P is divided by 2 x 31 = 62; likewise, the number of micrograms of ATP phosphorus must be divided by 3 x 31 = 93 to obtain the micromolar concentration of the nucleotide.

REFERENCES

Berenblum, I., and Chain, E. (1938). An improved method for the colorimetric determination of phosphate. Biochem. J. 32, 295-298.

Berg, H. (1937). Uber den Herzmuskelstoffwechsel bei Hyperthyreose und seine Beeinflussung durch Vitamin C. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 185, 359-367.

Bertolini, A.M., and Quarto di Palo, F. (1956). Effetto della tiroxina sugli esteri fosforici ad alto potenziale di energia nel cuore di ratto. Boll. soc. ital. biol. sper. 32, 91-93.

Bronk, J.R. (1958). Some effects of thyroxine on oxidative phosphorylation in submitochondrial particles and intact mitochondria. Biochim. Biophys. Acta 27, 667-668.

Chance, B. (1959a). Quantitative aspects of the control of oxygen utilization. In: Ciba Found. Symp. Regulation Cell Metab.,edited by G.E.W. Wolstenholme and C.M. O'Connor. Boston: Little, Brown and Company, 1959, pp. 91-121.

Chance, B., and Hess, B. (1959b). Metabolic control mechanisms. III. Kinetics of oxygen utilization in ascites tumor cells. J. Biol. Chem. 234, 2416-2420.

Chance, B., and Hess, B. (1959c). Metabolic control mechanisms. IV. The effect of glucose upon steady state of respiratory enzymes in the ascites cell. J. Biol. Chem. 234, 2421-2427.

Chatagner, F., and Gautheron, D. (1960). Influence des hormones thyroidiennes sur la teneur en adenosine triphosphate du foie du rat. Biochim. Biophys. Acta 41, 544-545.

Chilson, O.P., and Sacks, J. (1959). Effect of hyper-thyroidism on distribution of adenosine phosphates and glycogen in liver. Proc. Soc. Exptl. Biol. Med. 101, 331-332.

Davies, R.E. et. al. (1959).The energy supply for muscle contraction. Ann. New York Acad. Sc. 81, 468-480.

Fawaz, G., and Manoukian, E. (1962). Steady-state level of phosphocreatine in the heart. Circulation Research 11, 115-118.

Fawaz, G., and Tutunji, B. (1957). The mechanism of dinitrophenol heart failure. Brit. J. Pharmacol. 12, 273-280.

Feinstein, M. (1960). Ph.D. thesis, Department of Pharmacology, State University of New York, Downstate Medical Center, 1960. Cited in Furchgott and Lee (1961).

Fleckenstein, A. (1955). Der Kalium-Natrium-Austausch als Energieprinzip in Muskel und Nerv. Berlin, Göttingen, Heidelberg. Springer, 1955.

Fleckenstein, A. et al. (1954a). Chemistry of muscle contraction. Contraction of muscle without fission of adenosine triphosphate or creatine phosphate. Nature 174, 1081-1083.

Fleckenstein, A. et al. (1954b). Zerfällt Adenosintriphosphat bei der Muskelkontraktion? Pflügers Arch. ges. Physiol. 259, 246-259.

Fleckenstein, A. et al. (1959). Konzentration und Turnover der energiereichen Phosphate des Herzens nach Studien mit Papierchromatographie und Radiophosphor. Klin. Wschr. 8, 451-459.

Fleckenstein, A. et al. (1960). Die Inkorporation von markiertem Sauerstoff aus Wasser in die ATP-, Kreatinphosphat- und Orthophosphat-Fraktion intakter Muskeln bei Ruhe, tetanischer Reizung und Erholung. Pflügers Arch. ges. Physiol. 271, 75-104.

Fleckenstein, A., und Janke, J. (1953). Papierchromatographische Trennung von ATP, ADP und anderen Phosphor-Verbindungen im kontrahierten und erschlafften Froschmuskel. Pflügers Arch. ges. Physiol. 258, 177-199.

Furchgott, R.F., and de Gubareff, T. (1958). The high energy phosphate content of cardiac muscle under various experimental conditions which alter contractile strength. J. Pharmacol. and Exper. Therap. 124, 203-218.

Furchgott, R.F., and Lee, K.S. (1961). High energy phosphates and the force of contraction of cardiac muscle. Circulation 24, 416-428.

Gerlach, E., und Janke, J. (1958). Papierchromatographische Bestimmung von Kreatinphosphat und Orthophosphat in Gewebsextrakten. Biochem. Z. 330, 565-575.

Gerlach, E., und Lübben, K. (1959). Der Phosphat-Stoffwechsel in Tauben- und Menschen- Erythrocyten unter dem Einfluss von 2,4-Dinitrophenol, Natriumcyanid, Monojodacetat sowie von Thyroxin und Triäthylenmelamin. Pflügers Arch. ges. Physiol. 269, 520-554.

Hanes, C.S., and Isherwood, F.A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. Nature 164, 1107-1112.

Hoch, F.L. (1962). Biochemical actions of thyroid hormones. Physiol. Revs. 42, 605-673.

Hochrein, H., und Döring, H.J. (1958). Das Verhalten der energiereichen Phosphat-Verbindungen des Myokards im Normalzustand und bei experimenteller Insuffizienz durch Phenylbutazon. Pflügers Arch. ges. Physiol. 267, 313-330.

Holland, W.C. and Klein, R.L. (1960). The Chemistry of Heart Failure. Springfield, Ill. Charles C. Thomas, 1960.

Lardy, H.A., and Maley, G.F. (1954). Metabolic effects of thyroid hormones in vitro. Recent Prog. Hormone Res. 10, 129-155.

Lee, K.S. et al. (1960a). The effect of ouabain on the oxygen consumption, the high energy phosphates and the contractility of the cat papillary muscle. J. Pharmacol. and Exper. Therap. 129, 115-122.

Lee, K.S. et al. (1960b). An effect of cardiac glycosides on oxidative phosphorylation by heart mitochondria. J. Pharmacol. and Exper. Therap. 129, 123-127.

Lee, K.S., et al. (1961c). The influence of potassium and calcium on the effect of ouabain on cat papillary muscles. J. Pharmacol. and Exper. Therap. 132, 139-148.

Lohmann, K. (1928). Über das Vorkommen und den Umsatz von Pyrophosphat in Zellen. Biochem. Z. 202, 466-493.

Lohmann, K. (1934). Über die enzymatische Aufspaltung der Kreatinphosphorsäure; zugleich ein Beitrag zum Chemismus der Muskelkontraktion. Biochem. Z. 271, 264-277.

Markham, R., and Smith, J.D. (1949). Chromatographic studies of nucleic acids. A technique for the identification and estimation of purine and pyrimidine bases, nucleotides, and related substances. Biochem. J. 45, 294-298.

Mattonet, C. (1933). Chemischer Beitrag zur Frage der Herzmuskelschädigung durch Thyroxin. Z. ges. exp. Med. 90, 237-244.

Michal, G., and Lamprecht, W. (1961). Methoden zur Bestimmung von Metaboliten des Kohlenhydratstoffwechsels im Herzmuskel. Z. physiol. Chem. 324, 170-187.

Mommaerts, W.F.H.M. (1954). Chemistry of muscle contraction. Is adenosine triphosphate broken down during a single muscle twitch? Nature 174, 1083-1084.

Mommaerts, W.F.H.M. (1955). Investigation of the presumed breakdown of adenosine triphosphate during a single muscle twitch. Am. J. Physiol. 182, 585-593.

Mommaerts, W.F.H.M. (1961). The regulation of metabolism and energy release in contracting muscle. Circulation 24, 410-415.

Olson, R.E. et al. (1961). Cardiac myosin and congestive heart failure in the dog. Circulation 24, 471-482.

Olson, R.E. and Piatneck, D.A. (1959). Conservation of energy in cardiac muscle. Ann. New York Acad. Sc. 72, 466-471.

Schumann, H. (1939). Experimentelle Befunde zur Frage gesteigerter Thyroxinempfindlichkeit des mechanisch vermehrt belasteten Herzmuskels. Z. ges. exp. Med. 105, 577-583.

Wollenberger, A. (1947). On the energy-rich phosphate supply of the failing heart. Am. J. Physiol. 150, 733-745.

Wollenberger, A., et al. (1958). Orthophosphat- und Phosphokreatingehalt des Herzmuskels. Naturwissenschaften 45, 294.

Wollenberger, A. et al. (1960a). Eine einfache Technik der extrem schnellen Abkühlung grösserer Gewebestücke. Pflügers Arch. ges. Physiol. 270, 399-412.

Wollenberger, A. et al. (1960b). Über den tatsächlichen Orthophosphat- und N-Phosphorylkreatingehalt des Säugetierherzens. Pflügers Arch. ges. Physiol. 270, 413-427.

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